Enteral nutrients potentiate the intestinotrophic action of glucagon-like peptide-2 in association with increased insulin-like growth factor-I responses in rats

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Running head: GLP-2 induces mucosal growth and IGF-I responses

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ABSTRACT

Glucagon-like peptide-2 (GLP-2) is a nutrient-dependent, intestinotrophic hormone derived from posttranslational processing of proglucagon in the distal bowel. GLP-2 is thought to act through indirect mediators, such as insulin-like growth factor I (IGF-I). We investigated if intestinal expression of GLP-2 and IGF-I system components are increased with the mucosal growth induced by enteral nutrient (EN) and/or a low dose of GLP-2 in parentally-fed rats. Rats were randomized to 4 treatment groups using a 2×2 design and maintained with parental nutrition (PN) for 7 days: PN alone, EN, GLP-2, EN+GLP-2, n=7-9. The 2 main treatment effects are: ±GLP-2 (100 μg/kg body wt/day) and ±EN (43% of energy needs, day4-6). Combination treatment with EN+GLP-2 induced synergistic intestinal growth in ileum resulting in greater mucosal cellularity, sucrase segmental activity and gain of body weight (EN×GLP-2, p<0.04). In addition, EN+GLP-2 induced a significant 28% increase in plasma concentration of bioactive GLP-2, a significant 102% increase in ileal proglucagon mRNA with no change in ileal dipeptidyl peptidase-IV (DPP-IV) specific activity, and significantly reduced plasma DPP-IV activity compared to GLP-2. This indicates that EN potentiates the intestinotrophic action of GLP-2. Proliferation of enterocytes due to GLP-2 infusion was associated with greater expression of ileal proglucagon, GLP-2 receptor, IGF-I, IGF binding protein-3 mRNAs, and greater IGF-I peptide concentration in ileum (p<0.032). Ileal IGF-I mRNA was positively correlated with expression of proglucagon, GLP-2R, and IGFBP-5 mRNAs (R²=0.43-0.56, p<0.0001). Our findings support the hypothesis that IGF-I is one of the downstream mediators of GLP-2 action in a physiological model of intestinal growth.
Key words: parenteral nutrition, proglucagon, IGF binding protein-3 and -5, GLP-2 receptor
INTRODUCTION

GLP-2 is a potent intestinotropic hormone derived from tissue specific posttranslational processing of proglucagon in the endocrine L cells of the ileum and colon (13). GLP-2 is secreted in response to nutrient intake and is rapidly inactivated by dipeptidyl peptidase-IV (DPP-IV) with a biological half-life of ~7 min (10). Previous studies indicate that GLP-2 is a key mediator of intestinal adaptive growth through stimulation of epithelial cell proliferation and inhibition of apoptosis in a variety of models of intestinal dysfunction in mice, rats and pigs (1, 6, 11). Moreover, exogenous GLP-2 and a GLP-2 analogue resistant to degradation by DPP-IV improve intestinal morphology and energy absorption in humans with short bowel syndrome (16, 17). Given the therapeutic potential for use of GLP-2 in humans with compromised intestinal function, there is great interest in understanding the mechanisms by which GLP-2 regulates intestinal epithelial growth. Expression of the GLP-2 receptor (GLP-2R), a G protein-coupled receptor, occurs primarily in the gastrointestinal tract and central nervous system, with limited expression in lung, cervix and vagal afferents (24, 27). Within the intestine the GLP-2R has been localized to enteroendocrine cells, enteric neurons and subepithelial myofibroblasts (10, 27). In contrast, the target cells for intestinal growth, crypt epithelial cells and enterocytes, do not express the GLP-2R. This has led to the hypothesis that GLP-2 action requires downstream mediators to induce intestinal growth and regulate other aspects of intestinal physiology (10).

Insulin-like growth factor-I (IGF-I) has been identified as a potential downstream mediator of the intestinal growth effects of GLP-2. Recent work in mice showed that exogenous GLP-2 improves gut morphology and increases proliferative indices in wild type,
but not IGF-I knockout mice, suggesting an essential role for IGF-I in the intestinotrophic action of GLP-2 (11). Moreover, administration of either exogenous IGF-I or GLP-2 reverses the mucosal atrophy induced by parenteral nutrition in rodents (4, 7). IGF-I is a growth hormone-dependent anabolic hormone that stimulates whole body and intestinal growth in response to nutritional status. IGF-I has been well characterized as a potent stimulator of intestinal growth via endocrine mechanisms associated with administration of IGF-I, as well as paracrine mechanisms associated with local synthesis of IGF-I in the intestine (34). The intestine synthesizes IGF-I in smooth muscle and subepithelial myofibroblasts, which expresses the GLP-2R (30), and epithelial IGF-I receptors are widespread in the intestine (28). Thus, GLP-2 may increase IGF-I synthesis in subepithelial myofibroblasts with IGF-I acting as the downstream mediator to promote the intestinal growth induced by GLP-2 (12).

Enteral nutrients (EN) provide the primary stimulus for intestinal growth both directly by providing energy and protein to enterocytes and indirectly by stimulating hormonal mediators such as GLP-2 and IGF-I. The parenteral nutrition (PN) rat model is ideal to study the intestinotrophic effects of nutrient regulated hormones such as GLP-2 and IGF-I because it provides precise control of nutrient intake and induces 40-50% mucosal atrophy without the systemic alterations induced by fasting (7). The objective of the current study was twofold. First, to determine if the synergistic intestinal growth response to combination treatment with EN and a low-dose of GLP-2 occurs during the condition of PN-induced mucosal atrophy without the trophic stimulus of intestinal resection (21). Second, to further understanding of the hypothesis that IGF-I is one of the downstream mediators of GLP-2 action by
characterizing intestinal expression of GLP-2 and IGF-I system components that occur in conjunction with the intestinal growth induced by EN and GLP-2.

**MATERIALS AND METHODS**

*Animals and Experimental Design*

The animal facilities and research protocols were approved by the University of Wisconsin Madison Institutional Animal Care and Use Committee. Male, Sprague-Dawley rats (Harlan, Madison, WI) initially weighing 175-200 g were housed in individual, stainless steel cages with unlimited access to water in a room maintained at 22°C on a 12:12-h light-dark cycle. All rats were acclimated to the facility for 6 days while being fed a semi-purified diet ad libitum (7).

Rats were randomly assigned to four PN treatment groups (n=9) using a 2×2 factorial design as follows: PN alone, EN, GLP-2, and EN+GLP-2. Rats in these 4 groups were maintained with PN for 7 days. The two main treatment effects are: ±EN (days 4-6) and ±GLP-2 (100 µg/kg body wt/day). The final sample size for each group was: PN alone, n=7; EN, n=9; GLP-2, n=8; and EN+GLP-2, n=9. A non-surgical group of rats fed a semi-purified diet ad libitum was included for reference (Oral, n=9).

Rats were fasted for 18 h prior to surgical placement of intravenous catheters. Rats were anesthetized by inhalation of isofluorane (IsoFlo; Abbot Laboratories, North Chicago, IL) via an anesthesia machine before surgery. Intravenous catheters were placed in the superior vena cava as previously reported (7, 20). Infusion of PN solution was initiated using a Harvard syringe pump (Harvard apparatus, INC. Holliston, MA) at 1.0 ml/h immediately following surgery (day 0), advanced to 1.67 ml/h on day 1, and maintained at full strength
infusion of 2.5 ml/h (60 ml/day) for groups not receiving EN for days 2-6. Rats given PN alone and GLP-2 on days 2-6 received the following daily nutrient intake: 64 kcal, 2.6 g protein (16.5% energy), 1.7 g fat (24% energy), and 11 g dextrose (59% energy). The composition and preparation of the nutritionally complete PN solution was previously reported (7). Treatment groups given oral EN received 2.5 ml/h PN solution for days 2-4 and then the infusion was gradually decreased to 1.67 ml/hr on day 5 and 1.0 ml/h on day 6. A low-residue, semi-elemental liquid diet containing intact and hydrolyzed protein, safflower oil, medium chain triglycerides, sucrose and maltodextrins (Vital®, donated by Ross Products Division, Abbott Laboratories, Columbus, OH) was offered ad libitum in graduated feeding tubes on days 4-6, providing 1 kcal per ml with 16.7% energy from protein, 9.5% energy from fat and 73.8% energy from carbohydrate. Thus, approximately 43% of energy needs were provided by EN and 57% were provided by PN during the last three days of the study for EN and EN+GLP-2 groups.

Rats infused with GLP-2 received 100 µg human GLP-2/kg body wt/day concurrent with continuous intravenous infusion of PN solution for days 1-7. Human GLP-2 (preproglucagon 126-158, California Peptide Research, Inc, Napa, CA) was diluted in phosphate buffered saline, pH=7.4 one day before surgery and added to the PN solution daily. Vehicle was infused in rats not given GLP-2 (21). Assay of bioactive GLP-2 in the residual PN solution after daily infusion indicated greater than 95% recovery.

Body weights, PN solution infused and the amount of EN consumed were recorded daily. Urine was collected into containers with 0.1% boric acid for determination of nitrogen
balance (35). After 7 days of PN rats were anesthetized with isofluorane and killed by exsanguination.

**Intestinal Composition, Histology, Proliferation and Sucrase Activity**

Rats received an intravenous bolus of 0.2 mg/g body wt of Bromo-2'-deoxyuridine (BrdU; Sigma Chemical, St. Louis, MO) to label enterocytes for measuring proliferation at one hour before euthanasia. Rats were anesthetized and killed by cardiac exsanguination within 10 minutes of stopping the PN infusion. The entire small and large bowel and liver were removed for analysis. The bowel was sectioned into duodenum, defined as pylorus to ligament of Treitz; jejunum, defined as ligament of Treitz to ileum; ileum, defined as the final 25 cm of small bowel proximal to cecum, and colon. All sections of bowel were immediately flushed with ice-cold saline and put on a chilled glass plate to be sectioned. The first 3 cm of duodenum and colon and from 6-9 cm of jejunum and ileum were used for determining mucosal dry mass. The next 1 cm was fixed in 10% buffered formalin for histology and the next 3 cm were collected for determination of protein (bicinchoninic acid protein assay, Pierce Chemicals, Rockford, IL), DNA (19) and sucrase activity in jejunum (5). The first 5 cm and the remaining tissue of jejunum and ileum, were snap-frozen intact in liquid nitrogen and stored at –70°C for RNA extraction and determination of IGF-I content by RIA, respectively. Fixed tissue for histology was paraffin embedded, cut into 5-μm sections and processed for immunoperoxidase staining of BrdU-labeled cells and stained with hematoxylin and eosin for histomorphology as previously described (8). For each rat 10-12 crypts were counted.
Biochemical Analyses


Blood was collected in chilled tubes containing a final concentration of 1 mg/ml EDTA, 0.1 mM Diprotin A (MP Biomedicals, Aurora, OH), and 0.01 mM aprotinin (Calbiochem, La Jolla, CA). Plasma was isolated by centrifugation at 1,800 G for 15 min at 4°C and was stored at –70°C. Plasma bioactive GLP-2 was measured by RIA using an antibody that has an absolute requirement for the intact N terminal amino acid sequence of GLP-2 that confers its bioactivity, i.e., GLP-2, 1-33 (15). Plasma IGF-I was measured by RIA after removing binding proteins by HPLC under acidic conditions; the recovery of IGF-I was 85-90% (29).

Intact jejunum, and ileum samples were homogenized in ammonium formate, spun at 14,000 G for 15 min, and the supernatant was applied to a C-2 bond elute column (Varian, Harbor City, CA), as previously described (14). Immunoreactive IGF-I was extracted in 45% acetonitrile-3% trifluoroacetic acid and IGF-I concentration was determined by RIA (14, 29).

Dipeptidyl peptidase - IV activity. DPP-IV activity was measured in plasma and homogenates of ileal mucosa and intact colon using the discontinuous direct photometric method of Nagatsu et al. (25). The assay mixture included 5 µl of sample and 25 µl of 0.15 M glycine / NaOH pH 8.7 buffer. Substrate (25 µl of 3 mM glycylnproline-p-nitroanilide tosylate) was quickly added to the wells and plates were incubated at 37°C for 30 minutes. The reaction was stopped by rapidly adding 150 µl of 1M sodium acetate, pH 4.2. The amount of p-nitroaniline liberated per minute was used to determine enzyme activity based on the standard curve that contained 0-50 nmol p-nitroaniline. One unit of DPP-IV activity is defined as the amount of enzyme that hydrolyses 1 µmol of substrate per min. Specific
activity of DPP-IV in ileum and colon was expressed as unit of DPP-IV activity per g of protein.

*IGF-I, IGF binding protein-3 and -5, GLP-2R and Proglucagon mRNA.* Total RNA was extracted from intact jejunum and ileum using TRIzol reagent (GIBCO-BRL Life Technologies, Grand Island, NY) and quantified spectrophotometrically at 260 nm. Integrity was confirmed by visualization of 18S and 28S rRNA on an agarose-formaldehyde gel with ethidium bromide staining. IGF-I, IGF binding protein-3 (IGFBP-3) and IGFBP-5 mRNAs were measured by RNase protection assay (RPA) using a HybSpeed RPA kit (Ambion, Austin, TX) according to the procedure described previously (14). Quantification of proglucagon mRNA was done using a Northern Max kit (Ambion, Austin, TX) (6). All bands were quantified by light densitometry using OptiQuant software package (Packard Instruments, Meriden, CT). GLP-2R, IGF-I and proglucagon mRNA expression were also measured in a 2-step Reverse Transcriptase - Real Time PCR (RT-qPCR) using SYBR Green detection method as described previously (26). Sequences for forward and reverse primers (Integrated DNA Technologies, INC. Coralville, IA) are shown in Table 1. Data were analyzed using 7000 system software (Applied Biosystems) and relative quantification was done using $\Delta\Delta C_t$ method (22) with 18S as the internal control and the oral group as reference control.

*Statistical Analyses*

Treatment groups were analyzed using general linear models and individual differences between the treatment groups were identified by one-way ANOVA followed by the protected least significant-differences technique (SAS version 8.2; SAS Institute, Cary,
NC). Main effects of EN, GLP-2 and their interaction were assessed using two-way ANOVA. Statistics were performed on log-transformed data for data showing unequal variance among groups. All data are presented as means ± SE. P < 0.05 was considered statistically significant.

RESULTS

Body Weight and Nitrogen Balance

There were no significant differences in body weights among the groups before surgery, Figure 1. There were no significant differences in energy intake, body weight and nitrogen retention among the treatment groups prior to the introduction of EN (day 0-day 4).

During the period when rats received both PN and EN (day 4 to day 7), neither EN nor GLP-2 had a significant effect on change in body weight. However in the same period, combination treatment with EN+GLP-2 induced significantly greater gain in body weight (Time×EN×GLP-2, p=0.037). Total energy intake was significantly greater in the two groups receiving EN on day 4 (96±4; kcal/d) compared to the two groups who received only PN (64 kcal/d), but not significantly different on day 5 and 6. Total energy intake was not significantly different in the two groups given EN for days 4-6. Thus, in spite of similar energy intake, rats given combination treatment with EN+GLP-2 showed significantly greater gain in body weight, although differences in fluid retention cannot be ruled out. Liver mass was significantly greater in rats given EN or GLP-2 treatment compared with PN alone.
Mucosal Adaptive Growth

Rats given PN alone exhibited significant mucosal atrophy in the duodenum, jejunum and ileum, but not in the colon compared to oral reference, Figure 2. Data for duodenum and colon are not shown. Mucosal atrophy in the small bowel was not altered with individual treatment with EN such that mucosal dry mass, and concentrations of protein and DNA were not significantly different compared to PN alone. However, individual treatment with GLP-2 reversed the PN-induced mucosal atrophy in all sections of the small bowel such that mucosal cellularity was similar or greater compared with the oral reference.

The significant mucosa growth induced by GLP-2 was most pronounced in the jejunum and ileum. GLP-2 or EN+GLP-2 induced greater jejunum and ileum mucosal dry mass, and concentrations of protein and DNA by 34%-132% or 67%-192%, respectively, compared with PN alone, Figure 2. Indices of mucosal growth in the ileum showed significant interaction (EN×GLP-2, p<0.03) indicating a synergistic effect of treatment with EN+GLP-2 to further enhance mucosal adaptive growth beyond that expected by additive effects of the two treatments in ileum. Treatments with GLP-2 resulted in a significantly greater (p<0.001) ratio of protein to DNA in jejunal and ileal mucosa suggesting that the greater mass of jejenum and ileum were due to greater cell size and cell number compared to PN alone.

The histology data for jejunum and ileum was consistent with changes in mucosal cellularity. Individual treatment with EN did not alter jejunum and ileum villus height and crypt depth compared to PN alone, Table 2. Individual treatment with GLP-2 resulted in significantly greater jejenum and ileum villus height and crypt depth compared to PN alone.
The synergistic effect of combination treatment with EN+GLP-2 was noted for ileum villus height (p=0.0132), but not for crypt depth.

Enterocyte proliferation was measured with incorporation of BrdU by crypt cells, Table 2. The number of cells in a crypt column was significantly greater in both jejunum and ileum in animals treated with GLP-2 compared to the two groups not given GLP-2 (main effect, p<0.0001). The number of BrdU-labeled cells was significantly greater in animals treated with GLP-2 in ileum (main effect, p=0.0008) but not in jejunum. In summary, GLP-2 treatment increased crypt cell proliferation resulting in a greater total crypt cell population.

**Sucrase Activity**

Mucosal sucrase activity reflects the digestive capacity of jejunum. Individual treatment with EN or GLP-2 resulted in significantly greater jejunal sucrase segmental activity compared to PN alone, Figure 3. Combination treatment with EN+GLP-2 induced significantly greater sucrase segmental activity compared to either treatment alone (EN×GLP-2 interaction, p=0.0397). When sucrase activity was expressed as specific activity, individual treatment with EN or combination treatment with EN+GLP-2 induced significantly greater sucrase activity (EN, p=0.002). However, individual treatment with GLP-2 did not alter sucrase specific activity compared to PN alone, consistent with a population of immature enterocytes in the rats given GLP-2 without EN.

**Plasma Bioactive GLP-2 and DPP-IV activity**

Individual treatment with GLP-2 induced a significantly greater concentration of bioactive GLP-2 in plasma than did treatment with EN or PN alone, Figure 4. The plasma concentration of bioactive GLP-2 due to combination treatment with EN and GLP-2 was
significantly greater by 28% compared to GLP-2 alone. There were no significant differences in DPP-IV activity in plasma among the individual treatment groups with the exception of the EN+GLP-2 group. Combination treatment with EN and GLP-2 significantly reduced plasma DPP-IV activity by 75% compared to GLP-2 alone (EN×GLP-2 interaction, p=0.031). No significant main effect of EN, GLP-2 and their interaction was found in DPP-IV specific activity in ileum and colon, data not shown.

**Proglucagon mRNA and GLP-2 Receptor mRNA expression**

Ileum and colon are the primary sites for proglucagon expression, the precursor for GLP-2. Individual treatment with EN or GLP-2 significantly increased proglucagon mRNA expression in ileum, **Figure 5**. Ileal proglucagon mRNA expression due to combination treatment with EN and GLP-2 was 102% greater compared to GLP-2 alone (EN×GLP-2 interaction, p<0.045). In colon, EN and GLP-2 showed significant main effects to increase proglucagon mRNA expression without significant interaction (for EN, p=0.0015, for GLP-2 p=0.0303; data not shown).

Individual treatment with EN did not significantly alter the expression of GLP-2R mRNA in jejunum and ileum. Interestingly, individual treatment with GLP-2 and combination treatment with EN + GLP-2 induced a significantly greater expression of GLP-2R mRNA in ileum compared to PN alone (PN alone, 0.86±0.19 b; EN, 1.25±0.17 ab; GLP-2, 1.89±0.37 a; EN+GLP-2, 1.60±0.25 a; fold change compare to oral reference; GLP-2 main effect, p=0.0171). GLP-2 treatment did not significantly alter GLP-2R mRNA expression in jejunum.

**IGF-I responses**
The concentration of IGF-I in plasma was not significantly different between treatment groups (PN alone, 34±8; EN, 41±6; GLP-2, 52±3; EN+GLP-2, 46±9; nmol/L). In conjunction with greater mucosa growth, treatment with GLP-2 and EN+GLP-2, but not individual treatment with EN, induced significantly greater IGF-I mRNA expression and IGF-I peptide concentration in ileum, Figure 6. In jejunum, significant main effects for both EN (p<0.025) and GLP-2 (p<0.045) to increase IGF-I mRNA expression and IGF-I peptide concentration were observed, Figure 7.

Expression of IGFBP-3 and IGFBP-5 was examined in jejunum and ileum in the same gel. Significant main effects for both EN (p=0.0007) and GLP-2 (p=0.0345) to induce greater IGFBP-5 mRNA expression in ileum were observed, Figure 8. Treatment with GLP-2 and EN+GLP-2 induced significantly greater IGFBP-3 mRNA expression in ileum compared to the PN alone and EN groups. No significant differences between treatment groups were observed in jejunum for IGFBP-3 and IGFBP-5 mRNA expression (data not shown).

Correlations between GLP-2 and IGF-I system components

We examined the correlations between IGF-I mRNA and GLP-2 system components in ileum, the primary site for GLP-2 secretion, using RT-qPCR. Significant positive correlations were observed between IGF-I mRNA and proglucagon mRNA expression ($R^2 = 0.43$, p< 0.0001), IGF-I mRNA and GLP-2R mRNA expression ($R^2 = 0.47$, p< 0.0001), and proglucagon mRNA and GLP-2R mRNA expression ($R^2 = 0.42$, p=0.0003). In addition, we observed a positive correlation between IGF-I mRNA expression and IGFBP-5 mRNA expression using RPA ($R^2 = 0.56$, p< 0.0001).
DISCUSSION

IGF-I and GLP-2 are nutrient-regulated hormones that mediate, in part, the intestinotrophic effects of EN (10, 26, 28). Moreover, the actions of IGF-I and GLP-2 may be interrelated as evidence from IGF-I knockout mice suggest that IGF-I is an essential mediator of the intestinotrophic actions of GLP-2 (11). The reversal of PN induced mucosal atrophy with GLP-2 administration in the current study was associated with greater expression of proglucagon and the GLP-2R, as well as IGF-I and IGFBP-3 mRNAs, and IGF-I peptide in ileum. This suggests that the mucosal growth induced by GLP-2 is associated with upregulation of the IGF-I system in intestine.

Treatment with the combination of EN+GLP-2 induced a synergistic increase in intestinal growth and sucrase segmental activity that was associated with significantly greater gain of body weight compared with EN, GLP-2 or PN alone (21). The GLP-2 receptor has only been identified in tissues found in the brain and the gastrointestinal tract (33). Moreover, several in vivo studies have shown that GLP-2 treatment does not induce changes in body weight or organ weight other than the intestine, which suggests that GLP-2 is an intestine-specific growth factor (1, 4, 32, 33). Using a larger dose of GLP-2 than that used in the present study (240 µg · kg body wt⁻¹ · day⁻¹), Martin et al (23) also noted that GLP-2 infusion reduced loss of body weight in PN rats with proximal bowel resection. Greater circulating IGF-I would improve whole body anabolism, however, plasma concentration of IGF-I was not different among the treatment groups. Thus, enhanced absorption of nutrients due to an enlarged mucosa mass, consistent with greater sucrase activity and the sucrose content of the diet, may have contributed to the greater gain of body weight in rats treated with EN+ GLP-2.
Treatment with EN or GLP-2 alone resulted in significantly greater sucrase segmental activity, although only EN stimulated an increase in sucrase specific activity. These differential changes in segmental and specific sucrase activity suggest varying effects on enterocyte functional activity due to treatment with either EN or GLP-2 as previously noted in our rat model of short bowel syndrome (21). Thus, the synergistic effect of EN+GLP-2 to improve digestive capacity and gain in body weight reflects the complementary dual actions of EN to stimulate enterocyte differentiation and GLP-2 to stimulate enterocyte proliferation.

GLP-2 is secreted primarily by the ileum in response to EN and is rapidly inactivated by DPP-IV cleavage and cleared by the kidney (10). Rats given EN+GLP-2 showed a significant 28% increase in concentration of plasma GLP-2, a significant 102% increase in ileum proglucagon mRNA expression, no change in ileal DPP-IV specific activity, and a significant 75% decrease in plasma DPP-IV activity compared with GLP-2 alone. This suggests that EN potentiate the intestinotrophic effects of exogenous GLP-2 by increasing GLP-2 synthesis and reducing its cleavage by DPP-IV. Although GLP-2 induces intestinal growth by both endocrine and paracrine mechanisms, ileum as a primary site for proglucagon synthesis may have a higher local concentration of GLP-2 and display more paracrine effects of GLP-2. This may explain, in part, why ileum showed a greater increase in proglucagon expression and subsequent mucosal growth in response to EN+GLP-2 compared with jejunum.

GLP-2 action is thought to depend on downstream mediators such as the IGF-I paracrine system because the GLP-2R is not expressed on epithelial cells (10). The presence of the GLP-2R on subepithelial myofibroblasts (30), a primary site for IGF-I synthesis,
provides a mechanism by which local GLP-2 may stimulate IGF-I synthesis, interaction with IGF-I receptors that are widespread in intestine, and promote epithelial cell proliferation (28). Consistent with IGF-I acting as a local downstream mediator of GLP-2, we found GLP-2 treatment significantly upregulated expression of GLP-2R mRNA as well as IGF-I and IGFBP-3 mRNAs, and IGF-I peptide concentration in ileum. Administration of IGF-I or increased local expression of IGF-I is known to increase expression of its carrier protein, IGFBP-3 (31, 34). This study confirms our report in resected rats that a low-dose of GLP-2 increases expression of the GLP-2R (18).

Treatment with EN+GLP-2 significantly increased both IGF-I and IGFBP-5 mRNA expression in ileum in association with synergistic ileal mucosal growth. IGFBP-5 is known to potentiate the intestinotrophic effects of IGF-I in PN and resected rats (14, 31), transgenic mice that overexpress IGF-I in mesenchyme (34), and cultured smooth muscle cells derived from intestine (3, 9). Williams et al (34) found that IGFBP-5 is specifically increased in the villus and pericryptal regions of the ileal lamina propria of mice that overexpress IGF-I in intestinal mesenchymal cells and these mice show preferential paracrine growth effects on the ileal mucosal epithelium. Taken together, the synergistic growth in ileum due to treatment with EN+GLP-2 and its association with increased local expression of IGF-I and IGFBP-5, as well as significant positive correlations between IGF-I, proglucagon and GLP-2R mRNA expression, support the hypothesis that IGF-I acts as a paracrine downstream mediator of GLP-2 in the current study.

EN alone did not show a significant effect to increase plasma concentration of GLP-2 and stimulate mucosal growth (2, 21, 26), in spite of the dramatic mucosal growth induced by
treatment with EN+GLP-2. This may reflect the small amount of EN provided, only 43% of energy needs during the last 3 days of the study, as well as the semi-elemental nature of the liquid diet. Studies in the neonatal pigs indicate that 60% of energy requirements from EN are required to sustain normal plasma concentration of GLP-2 and mucosal growth (2).

In conclusion, we demonstrate for the first time in rats with PN-induced mucosal atrophy that proliferation of enterocytes due to GLP-2 is associated with greater expression of ileal proglucagon, GLP-2R, IGF-I and IGFBP-3 mRNAs, as well as increased IGF-I peptide in ileum. Thus, our findings support the hypothesis that IGF-I is one of the downstream mediators of GLP-2 action in a physiological model of intestinal growth.

**PERSPECTIVES AND SIGNIFICANCE**

The primary physiologic stimulus for maintaining enterocyte turnover is the presence of nutrients in the gastrointestinal tract. Our data in the model of PN-induced mucosal atrophy show no effect of a small amount of EN alone to induce mucosal growth but a dramatic synergy between the presence of EN and administration of a low-dose of GLP-2 to promote epithelial cell proliferation and digestive capacity. These data emphasize the importance of encouraging EN in conditions where GLP-2 may be administered to humans with intestinal failure. The hypothesis that IGF-I is a downstream mediator of the intestinotrophic effects of GLP-2 is primarily based on a report where IGF-I knockout mice did not show growth in response to administration of GLP-2 (11). The current study provides strong evidence in a physiological model of intestinal atrophy and regrowth for this hypothesis, i.e., the intestinal paracrine IGF-I system may mediate the intestinotrophic effects
of GLP-2. Further studies are needed to understand the cellular mechanisms by which GLP-2 and IGF-I may interact to stimulate mucosal growth.

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GRANTS

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29. **Ney DM, Yang H, Smith SM, and Unterman TG.** High-calorie total parenteral nutrition reduces hepatic insulin-like growth factor-I mRNA and alters serum levels of


**Table 1** Primer sequences used in RT-qPCR

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<th>Target</th>
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RT-qPCR, 2-step Reverse Transcriptase - Real Time PCR; IGF-I, insulin-like growth factor I; GLP-2R, glucagon-like peptide 2 receptor.
Table 2. Histology, number of crypt cells and BrdU-labeled cells of jejunum and ileum in rats maintained with parenteral nutrition (PN) for 7 days including PN alone, EN, GLP-2 and EN+GLP-2.

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<th></th>
<th>PN alone (mm)</th>
<th>EN (mm)</th>
<th>GLP-2 (mm)</th>
<th>EN+GLP-2 (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Jejunum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Villus Height</td>
<td>0.46±0.02 b</td>
<td>0.49±0.02 b</td>
<td>0.78±0.02 a</td>
<td>0.81±0.03 a</td>
</tr>
<tr>
<td>Crypt Depth</td>
<td>0.11±0.003 b</td>
<td>0.11±0.005 b</td>
<td>0.16±0.005 a</td>
<td>0.15±0.007 a</td>
</tr>
<tr>
<td>No. of Crypt cells</td>
<td>24±1 b</td>
<td>24±1 b</td>
<td>35±1 a</td>
<td>35±1 a</td>
</tr>
<tr>
<td>No. of BrdU labeled cells</td>
<td>5±1 b</td>
<td>6±2</td>
<td>5±1</td>
<td>6±0</td>
</tr>
<tr>
<td><strong>Ileum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Villus Height</td>
<td>0.29±0.01 c</td>
<td>0.27±0.01 c</td>
<td>0.39±0.01 b</td>
<td>0.45±0.02 a</td>
</tr>
<tr>
<td>Crypt Depth</td>
<td>0.10±0.003 b</td>
<td>0.09±0.004 b</td>
<td>0.12±0.003 a</td>
<td>0.13±0.006 a</td>
</tr>
<tr>
<td>No. of Crypt cells</td>
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<td>23±1 c</td>
<td>31±1 a</td>
<td>28±1 b</td>
</tr>
<tr>
<td>No. of BrdU labeled cells</td>
<td>6±1 b</td>
<td>5±0 b</td>
<td>10±1 a</td>
<td>8±1 a</td>
</tr>
</tbody>
</table>

Values are means ± SE; n=7-9 for histology and n=3-5 for BrdU labeled cells. Values in the same row with different superscripts are significantly different (p< 0.05). BrdU, bromo-2'-deoxyuridine; PN, parenteral nutrition; EN, enteral nutrition; GLP-2, glucagon-like peptide 2.
Figure 1. Daily changes in body weight (g) and energy intake (kcal/day) in rats maintained with parenteral nutrition (PN) for 7 days as follows: PN alone, enteral nutrition (EN), glucagon-like peptide-2 (GLP-2), and EN+GLP-2. Rats in all groups showed similar gain of body weight during the period after surgery and before EN treatment, day 0 to day 4. During the period when rats received partial EN (day 4 to day 7), EN+GLP-2 induced significantly greater gain of body weight compared to other groups (Time×EN×GLP-2, p=0.037). *Significantly different final body weight or energy intake compared to other groups, p<0.05. Values are means + SE, n=7-9.

Figure 2. Jejunal and ileal mucosal dry mass (A), protein (B), and DNA (C) in rats fed orally or maintained with parental nutrition (PN) for 7 days as follows: PN alone, EN, GLP-2, and EN+GLP-2. Values are means + SE; n = 7-9. Means with different superscripts are significantly different (p< 0.05).

Figure 3. Jejunal mucosal sucrase segmental activity (A) and specific activity (B) in rats fed orally or maintained with PN for 7 days as follows: PN alone, EN, GLP-2 and EN+GLP-2. Values are means + SE; n =7-8. Means with different superscripts are significantly different (p< 0.05).

Figure 4. Plasma concentration of bioactive GLP-2 (A) and DPP-IV activity (B) in rats fed orally or maintained with PN for 7 days as follows: PN alone, EN, GLP-2 and EN+GLP-2. Values are means + SE. Sample size was n=7-9 for plasma concentration of bioactive GLP-
2. Sample size was n=5 for plasma DPP-IV activity except for the EN group where n=3.
Means with different superscripts are significantly different (p< 0.05).

**Figure 5.** Representative bands from Northern blot analysis of proglucagon mRNA and 18S rRNA (A) and ileal proglucagon mRNA expression (B) in rats maintained with PN for 7 days as follows: PN alone, EN, GLP-2 and EN+GLP-2 . Values are means + SE; n = 6-8. Means with different superscripts are significantly different (p< 0.05).

**Figure 6.** Representative bands from RNase protection assay of IGF-I mRNA (A), ileal IGF-I mRNA expression (B) and concentration of IGF-I peptide (C) in rats fed orally or maintained with PN for 7 days as follows: PN alone, EN, GLP-2 and EN+GLP-2. Ileal IGF-I mRNAs were determined by RNase protection assay. Thirty µg ileal RNA was hybridized with ³²P-labeled probes followed by RNase digestion and gel electrophoresis. Data was analyzed using OptiQuant software and expressed relative to 18S rRNA levels. Ileal IGF-I peptide concentration was determined by RIA. Values are means + SE; n = 7-9 for ileal IGF-I mRNAs and n = 3-5 for ileal IGF-I peptide concentration. Means with different superscripts are significantly different (p< 0.05).

**Figure 7.** Representative bands from RNase protection assay of IGF-I mRNA (A), jejunal IGF-I mRNA expression (B) and concentration of IGF-I peptide (C) in rats maintained with PN for 7 days as follows: PN alone, EN, GLP-2 and EN+GLP-2. Jejunal IGF-I mRNAs were determined by RNase protection assay. Thirty µg jejunal RNA was hybridized with
32P-labeled probes followed by RNase digestion and gel electrophoresis. Data was analyzed using OptiQuant software and expressed relative to 18S rRNA levels. Jejunal IGF-I peptide concentration was determined by RIA. Values are means + SE; n = 7-9 for jejunal IGF-I mRNAs and n = 3-5 for jejunal IGF-I peptide concentration. Means with different superscripts are significantly different (p< 0.05).

**Figure 8.** Representative bands from RNase protection assay of IGFBP-3 and IGFBP-5 mRNA (A), ileal IGFBP-3 mRNA (B) and IGFBP-5 mRNA (C) expression in rats maintained with PN for 7 days as follows: PN alone, EN, GLP-2 and EN+GLP-2. Ileal IGFBP-3 and IGFBP-5 mRNAs were determined by RNase protection assay in the same gel. Thirty µg ileal RNA was hybridized with 32P-labeled probes followed by RNase digestion and gel electrophoresis. Data was analyzed using OptiQuant software and expressed relative to 18S rRNA levels. Values are means + SE; n = 5-8. Means with different superscripts are significantly different (p< 0.05).
Figure 1

This figure illustrates the body weight and energy intake changes over 7 days across different diet treatments: PN alone, EN, EN+GLP-2, and GLP-2. The body weight (g) and energy intake (Kcal/day) are plotted over the days from 0 to 7.

- **Body Weight (g)**: The body weight is measured in grams and shows a gradual increase over the 7 days. The lines represent the different treatments, with PN alone showing the least increase, and EN+GLP-2 showing the most significant increase.

- **Energy Intake (Kcal/day)**: The energy intake is measured in Kcal/day and shows a peak on day 5, with the EN+GLP-2 treatment having a significantly higher intake compared to the others.

* indicates a statistically significant difference.
Figure 2

Dry Mass (mg/cm)

Protein (mg/cm)

DNA (mg/cm)

Oral
 PN alone EN GLP-2 EN+ GLP-2
 Parenteral Nutrition

Oral
 PN alone EN GLP-2 EN+ GLP-2
 Parenteral Nutrition
Figure 4

A

Plasma GLP-2 (pmol/L)

B

Plasma DPP-IV Activity (U/L)

Parental Nutrition

Oral  PN alone  EN  GLP-2  EN+ GLP-2

28%  75%

a  a  a  a  a  b  b
Figure 5

A
Proglucagon mRNA
18S

B
Bar graph showing Proglucagon : 18S levels under different conditions:
- PN alone
- EN
- GLP-2
- EN+GLP-2

Legend:
- c
- b
- a

Increased by 102%
Figure 6

A

Parental Nutrition

- + M PN alone EN GLP-2 EN+ GLP-2

IGF mRNA

18S

B

Ileum IGF-I mRNA :18S

0.00

0.03

0.06

0.09

0.12

0.00

0.03

0.06

0.09

0.12


C

Ileum IGF-I (ng/g)

0

30

60

90

120

150

PN alone EN GLP-2 EN+ GLP-2

Parental Nutrition

\( a \)

\( b \)
Figure 7

(A) Gel showing IGF-I and 18S mRNA levels under different nutritional conditions.

(B) Bar graph demonstrating the Jejunum IGF-I mRNA levels normalized to 18S mRNA, showing statistical differences (a, b).

(C) Bar graph showing Jejunum IGF-I levels (ng/g) under various nutrition conditions, with statistical differences (a, b).
Figure 8

**A**

Parental Nutrition

- + M
- PN alone
- EN
- GLP-2
- EN+ GLP-2

IGFBP3
IGFBP5
18S

**B**

<table>
<thead>
<tr>
<th>Ileum IGFBP-3 mRNA:18S</th>
</tr>
</thead>
<tbody>
<tr>
<td>b</td>
</tr>
<tr>
<td>b</td>
</tr>
<tr>
<td>a</td>
</tr>
<tr>
<td>a</td>
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</table>

**C**

<table>
<thead>
<tr>
<th>Ileum IGFBP-5 mRNA:18S</th>
</tr>
</thead>
<tbody>
<tr>
<td>b</td>
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<tr>
<td>ab</td>
</tr>
<tr>
<td>b</td>
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